

# Lignin engineering

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Lignins are aromatic polymers that are present mainly in secondarily thickened plant cell walls. Several decades of research have elucidated the main biosynthetic routes toward the monolignols and demonstrated that lignin amounts can be engineered and that plants can cope with large shifts in *p*-hydroxyphenyl/guaiacyl/syringyl (H/G/S) lignin compositional ratios. It has also become clear that lignins incorporate many more units than the three monolignols described in biochemistry textbooks. Together with the theory that lignin polymerization is under chemical control, observations hint at opportunities to design lignin structure to the needs of agriculture. An increasing number of examples illustrates that lignin engineering can improve the processing efficiency of plant biomass for pulping, forage digestibility and biofuels. Systems approaches, in which the plant's response to engineering of a single gene in the pathway is studied at the organismal level, are beginning to shed light on the interaction of lignin biosynthesis with other metabolic pathways and processes.

## Addresses

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## Introduction

Lignins are synthesized from the oxidative coupling of *p*-hydroxycinnamyl alcohol monomers and related compounds [1,2]. These polymers occur mainly in secondarily thickened plant cell walls. They are covalently bound to hemicelluloses and provide strength and rigidity to the cell wall, allowing plants to grow upward. They also provide the vascular system with the hydrophobicity needed for transport of water and solutes. Lignins have attracted significant research attention because they represent a major obstacle in chemical pulping, forage

digestibility, and processing of plant biomass to biofuels. These industries would benefit from processing biomass with either less lignin or a lignin that is easier to degrade.

In the past two decades, significant insight into the biosynthesis of lignin has been obtained by altering the expression of individual genes of the phenylpropanoid and monolignol biosynthetic pathway and studying the consequences on lignin amount and composition and on end-use properties [3]. This rather lignocentric analysis is now being extended to a more comprehensive phenotyping at the organismal level. This paper reviews new concepts and trends that have emerged from the lignin field over the past two years and that are of key importance in tailoring plant cell walls for end-use applications.

## Lignin monomer biosynthesis

The general picture that emerges from two decades of studies on the individual roles of the monolignol biosynthetic genes is that downregulation of *PAL*, *C4H*, *4CL*, *HCT*, *C3H*, *CCoAOMT*, *CCR*, and, to a lesser extent, *CAD*, have a prominent effect on lignin content (for abbreviations, see Figure 1) (Table 1) [1,3–7,8\*,9–13]. Lignin composition (H/G/S) can be engineered as well. *HCT*- and *C3H*-downregulated plants are strikingly enriched in H units, which are a minor component of typical wild-type lignin [5,9,14\*,15\*\*,16]. Downregulation of *F5H* results in lignin essentially composed of G units, whereas *F5H* overexpression can result in plants with lignins almost entirely composed of S units. *COMT* downregulation reduces S units and leads to the incorporation of 5-hydroxyconiferyl alcohol into the polymer. Finally, hydroxycinnamaldehydes are incorporated as a result of *CAD* downregulation, particularly into angiosperm lignin [1,3].

Reduced lignin contents are typically associated with dramatic changes in the soluble phenolic pools, and different species accumulate various storage and detoxification products [4,7,11,17\*,18]. When lignin levels become too low, plant growth and development is affected [7,10,11,16,17\*]. These pleiotropic growth defects have often been attributed to a dysfunctioning of the vascular system, but recent data show that this is not necessarily the case. For example, Besseau *et al.* [15\*\*] demonstrated that the growth reduction in *Arabidopsis thaliana* *het* mutants that deposit less, but H-unit-enriched lignin is caused by the overproduction of auxin transport-inhibiting flavonoids. Blocking flavonoid production in these plants by introducing a *chalcone synthase* (*chs*) mutation alleviates the dwarfing and restores a wild-type phenotype, but with H-rich lignin. Hence, plants appear

Table 1

**Effects on lignin content and H/G/S composition in various mutant and transgenic plants with altered monolignol biosynthesis relative to wild type**

Gene(s)	Total lignin	H	G	S	S/G	References
<i>PAL</i> ↓	↓	↓	↓	↓	↓/↑	[3,6,49]
<i>PAL</i> ↑	↑	n.a.	↑/No changes	↓/No changes	↓/No changes	[3]
<i>C4H</i> ↓	↓	↓	↓	↓	↓	[3,6]
<i>C4H</i> ↑	No changes	n.a.	No changes	No changes	No changes	[3]
<i>4CL</i> ↓	↓	↑	↓	↓	No changes	[3]
<i>HCT</i> ↓	↓	↑	↓	↓	↑	[6,13,15**]
<i>C3H</i> ↓	↓	↑	↓	↓	n.a.	[3,14*]
<i>CCoAOMT</i> ↓	↓	↑	↓	↓/No changes	↓/No changes/↑	[3,6,10]
<i>CCR</i> ↓	↓	↓	↓	↓	↓/↑	[3,7,17*]
<i>F5H</i> ↓	↓/No changes	n.a.	↑	↓	↓	[3,6]
<i>F5H</i> ↑	↓/No changes	n.a.	↓	↑	↑	[3]
<i>COMT</i> ↓	↓/No changes/↑	n.a.	↓/↑	↓	↓	[3,6,10]
<i>COMT</i> ↑	No changes	n.a.	No changes	No changes	No changes	[3]
<i>CAD</i> ↓	↓/No changes	n.a.	↑/No changes	↓/No changes	↓/No changes	[3,12]
<i>4CL</i> ↓ <i>F5H</i> ↓	↓	n.a.	n.a.	n.a.	↑	[3]
<i>CCoAOMT</i> ↓ <i>COMT</i> ↓	↓/No changes	n.a.	↓/No changes	↓	↓	[3,10]
<i>CCR</i> ↓ <i>COMT</i> ↓	↓	n.a.	n.a.	n.a.	↑	[3]
<i>CCR</i> ↓ <i>CAD</i> ↓	↓	n.a.	↓	↓	↑	[3]
<i>COMT</i> ↓ <i>CCR</i> ↓ <i>CAD</i> ↓	↓	n.a.	n.a.	n.a.	n.a.	[3]

The table summarizes the consequences of altering the expression of monolignol biosynthesis genes on lignin amount and composition assembled from numerous studies. As pathway fluxes might slightly differ among species, and as the degree of up/downregulation might differ between individual mutant and transgenic plants, and as the different plants were often analyzed with different analytical methods, opposing results were sometimes obtained. The abbreviations used for the genes are the same as in Figure 1; n.a., no data available.

to have the potential to cope rather well with H/G/S compositional shifts in the lignin polymer.

In addition, lignin clearly incorporates many more than the classical three monolignols *p*-coumaryl, coniferyl, and sinapyl alcohol (Figure 1). For example, products from incomplete monolignol biosynthesis, such as 5-hydroxyconiferyl alcohol [1], hydroxycinnamaldehydes [1,19], and hydroxycinnamic acids [17\*,20], or enzymatically made derivatives of the classical monolignols, such as sinapyl *p*-hydroxybenzoate [19,21], coniferyl and sinapyl *p*-coumarate, and coniferyl and sinapyl acetate [2,22\*] may all incorporate into the polymer at various levels. In some plants, such as abaca (*Musa textilis*), lignin acetylation even exceeds 80% of the uncondensed S units [22\*,23]. Together with the striking notion that the composition of the lignin polymer can be modified to either predominantly H, G, or S, these data demonstrate the extraordinary plasticity of the lignin polymerization process.

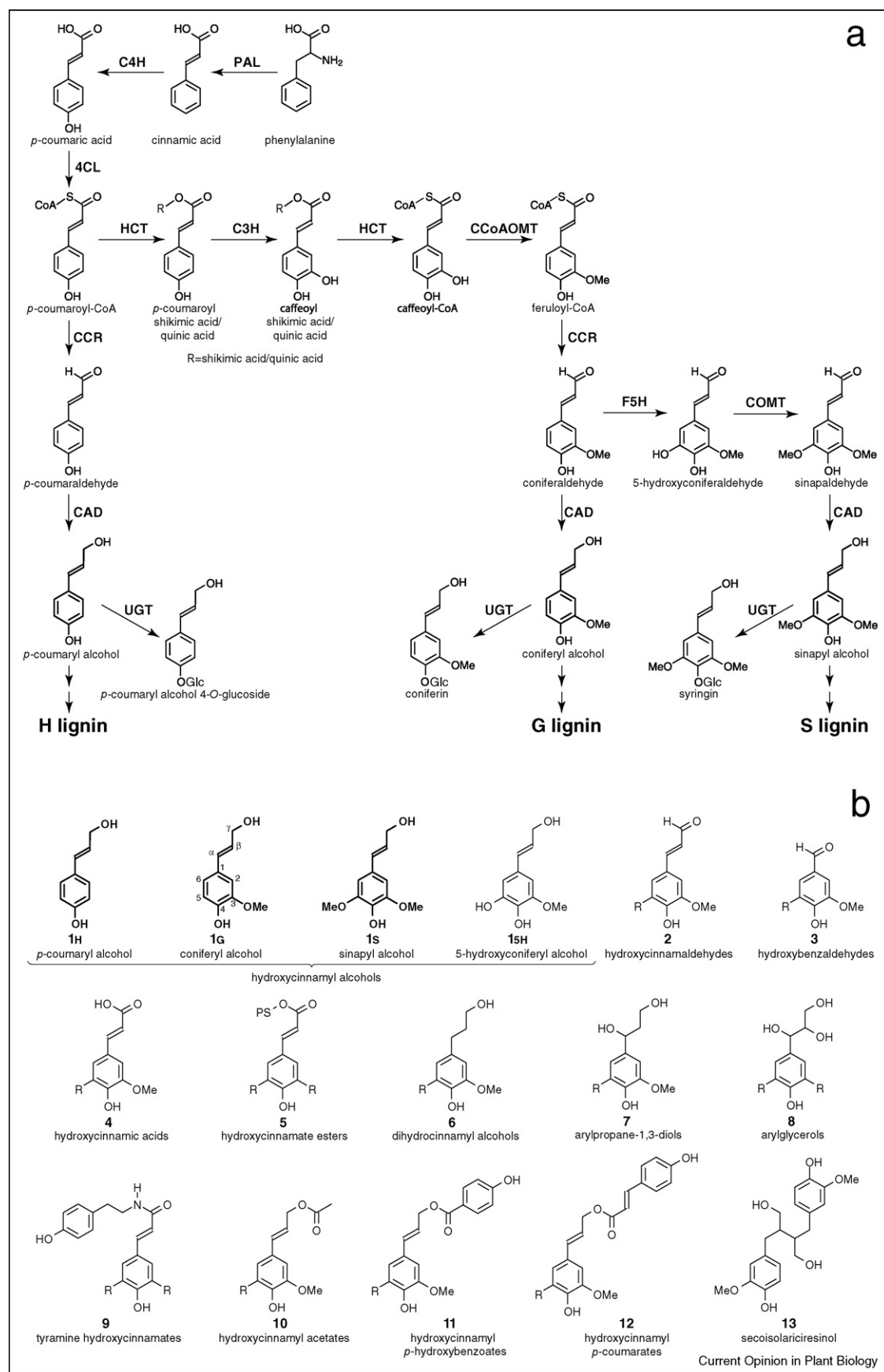
### Transport, coupling, and polymerization

After lignin monomers are biosynthesized, they are translocated to the cell wall, where they are oxidized for polymerization. The phenolic glucosides coniferin and syringin (Figure 1) have been considered as the transport forms of coniferyl and sinapyl alcohols before their aglycones are polymerized into lignin, but this has not been demonstrated yet. Genes have been cloned that encode coniferyl and sinapyl alcohol 4-*O*-glucosyltransferases [24] and cell-wall-localized  $\beta$ -glucosidases for coniferin

and syringin [25]. Transgenic Arabidopsis plants in which the expression of these glucosyltransferases is downregulated have severely reduced levels of the glucosides [26]. However, no significant lignin phenotype was observed (A Lanot, R Dixon, and D. Bowles, personal communication). Because coniferin and syringin do not accumulate to high levels in angiosperm xylem, and coniferyl and sinapyl alcohols might have the capacity to freely diffuse through the plasma membrane [27], these glucosides might play no role in monolignol export for developmental lignin. Accommodation of the multitude of alternative lignin monomers (Figure 1) might support a non-specific rather than a glucosyl transferase/ $\beta$ -glucosidase-mediated transport route.

The monolignols are oxidized by peroxidases or laccases [2,28–34]. The broad substrate specificities and the large gene families of these two classes of oxidases have made it difficult to identify isoforms that are specifically involved in developmental lignification. Co-expression analyses of genes expressed during stages of active lignification or during tension wood formation and gene-family-wide expression studies are beginning to associate individual gene family members with specific processes [35–38]. One member of the Arabidopsis laccase gene family has been proven, by genetic analysis, to be involved in lignin synthesis, albeit in the seed [39]. Different peroxidase isoforms typically have different kinetic properties *in vitro* prompting the question whether, and to what extent, these peroxidases help define the lignin composition, and thus structure, in the cell wall.

Figure 1



One of the most heated debates in lignin research is the opposing view of how monolignols couple during lignification. The theory originally developed by Freudenberg contends that lignin monomers are oxidized and then coupled in a combinatorial fashion. Because lignification is a chemical process, any phenol present in the lignifying zone of the cell wall is capable of entering into the combinatorial free radical-coupling process to the extent allowed by simple chemical concerns, such as structural compatibility, and influenced by typical physical parameters, such as pH, temperature, ionic strength, monolignol supply, hydrogen peroxide and peroxidase concentrations, and the matrix in general [5,19,28,40–42]. The influence of these parameters on lignin deposition is difficult to follow *in planta* but demonstrable in mimetic systems in which monolignols are polymerized under various conditions, such as the presence of polysaccharides [43,44–46]. A new hypothesis, however, surmises that the lignin monomers are coupled with absolute structural control by proteins bearing arrays of dirigent sites [47]. To date, however, there are no scientific observations that contradict the Freudenberg hypothesis but numerous against the dirigent model of lignin polymerization [48].

The presumed presence of a non-specific monolignol transport route, the broad substrate specificity of peroxidases, and the plasticity of the lignin polymerization process open the possibility of tailoring novel lignins composed of units that normally do not end up in the cell wall, and that make inter-unit bonds that are readily cleaved by lignin degradative processes. Acetal bonds might be just one such type (Figure 2). Such bonds were recently demonstrated for the first time by degradative and NMR structural analyses of lignins derived from *CCR*-deficient and wild-type plants that incorporate ferulic acid as a monomer [17,20].

### Systems biology

The analysis of lignin mutants by transcriptome and metabolome profiling has now made it clear that altering the expression of individual genes of the monolignol biosynthetic pathway has far-reaching consequences on plant metabolism. First, these deep phenotyping tools reveal regulation within the monolignol biosynthetic pathway itself, i.e., downregulation of one gene of the

monolignol pathway affects the transcript levels of other members of the same gene family, or other genes of the monolignol biosynthetic pathway [8,14,18,49–52].

Second, interactions between lignin metabolism and the biosynthesis of other cell wall polymers became apparent [12,14,18,49]. For example, *CCR*-downregulated transgenic poplars (*Populus* sp.) deposit less lignin, as expected from previous research on the monolignol biosynthetic pathway, but transcriptome and metabolome analyses also indicated a reduced biosynthesis of xylans, an observation that was not anticipated but further supported by wet chemistry [17]. Knowledge about these broader effects is essential to fully comprehend how gene function and cell wall properties are linked, how these cell wall properties are elaborated, and how they relate to the quality of raw material destined for agro-industrial uses.

Third, the systems biology approach reveals interactions of lignin biosynthesis with global metabolism, such as primary metabolism and stress pathways [17,49]. For example, downregulation of *CCR* in tobacco (*Nicotiana tabacum*) increases starch metabolism and photorespiration [18]. Insight into the plant's molecular response to lignin engineering might help mitigate these adverse effects in future engineering strategies, for instance by gene stacking [15,52].

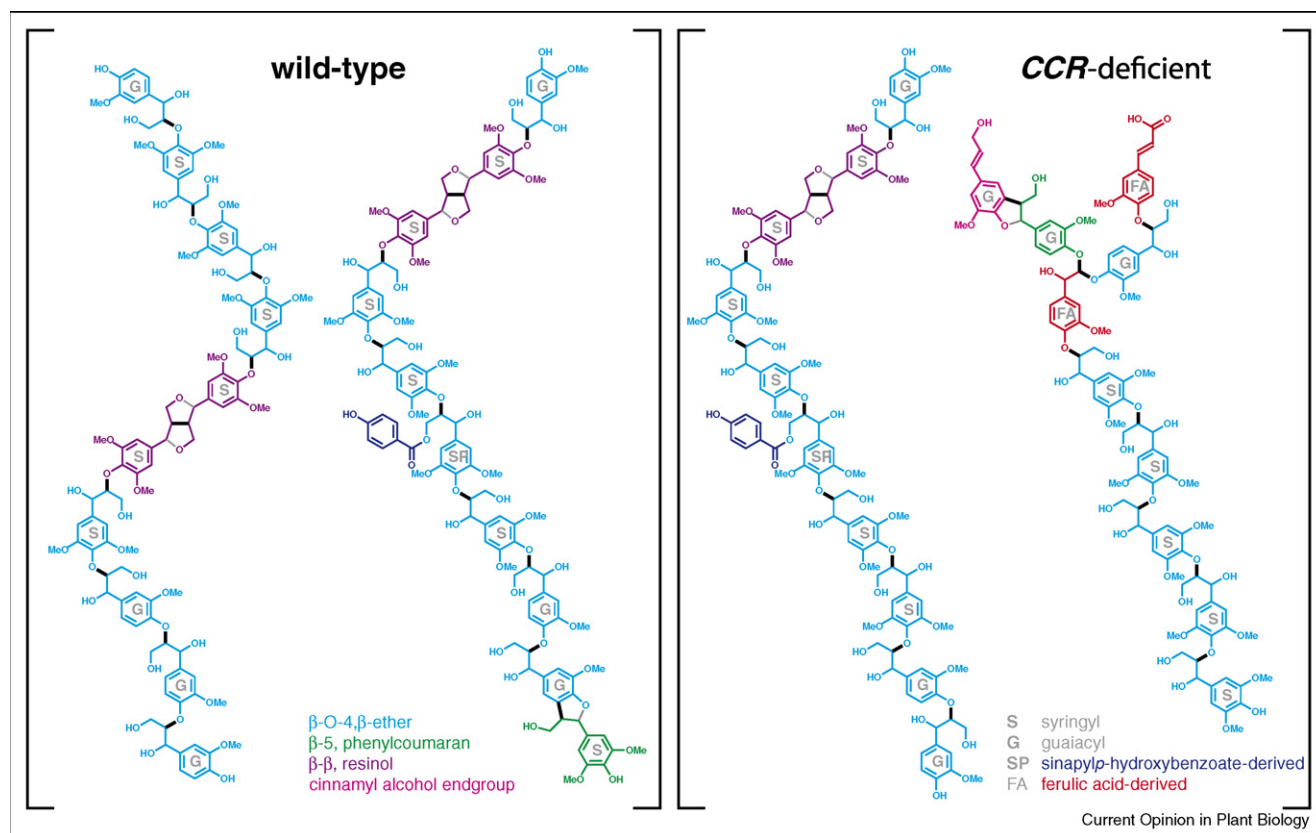
Fourth, transcript profiling of lignin mutants identifies new genes whose function is potentially closely associated with lignin biosynthesis. A growing list of candidate regulatory genes with a putative role in regulating monolignol biosynthesis has already been assembled by various transcript profiling experiments [12,18,35–37,53–55], but the number of confirmed regulators remains low. Nevertheless, several MYB and LIM family transcription factors have been shown to bind to monolignol biosynthetic promoters *in vitro* and to regulate these genes *in planta* [56–58].

### Translational research

Ultimately, our knowledge of lignin biosynthesis needs to be valorized to improve plant varieties for end-use applications, such as pulping, forage digestibility, or conversion to biofuels, either through genetic engineering or by exploiting natural variation. Several studies, some using

**(Figure 1 Legend)** (a) Monolignol biosynthetic pathway in angiosperms. Only the predominant route toward the three main monolignols is shown. Different species, cell types or conditions may have different fluxes through the pathway. For a more extensive pathway description and a discussion of the various routes, the reader is referred to references [1,2]. There are only a few reports on the presence of *p*-coumaryl alcohol-4-*O*-glucoside in plants. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; HCT, *p*-hydroxycinnamoyl-CoA: quinate shikimate *p*-hydroxycinnamoyltransferase; C3H, *p*-coumarate 3-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid/5-hydroxyconiferaldehyde *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; UGT, UDP-glucosyltransferase. (b) Lignin monomers (modified from reference [1]). Lignins derive primarily from the three monolignols 1, namely *p*-coumaryl alcohol 1H, coniferyl alcohol 1G, and sinapyl alcohol 1S (with the smaller descriptors indicating the type of aromatic nucleus, *p*-Hydroxyphenyl, Guaiacyl, or Syringyl, resulting from incorporating the monomer into the polymer). 15H is a monomer in *COMT*-deficient plants resulting in 5-hydroxyguaiacyl units in the form of benzodioxanes in the polymer. Other precursors 2–13 incorporate into lignins to varying degrees. PS, polysaccharide; R = H or OMe to define various *p*-hydroxyphenyl, guaiacyl or syringyl moieties.

Figure 2



Lignin polymer models for lignins from wild-type and *CCR*-deficient poplars. The models represent two decamer lignin chains with the main linkage types. The frequencies of the monomers and the inter-unit bonds are estimated from the NMR spectra of lignins isolated from wild-type (left) and *CCR*-deficient (right) poplars presented in Lep   et al. [17] and Ralph et al. [20] and are based on previous models [1,2]. The ferulate level is overrepresented but illustrates that ferulate incorporation results in a new (acetal) bond type and in branching of the polymer. Color coding is uniform across the two models. Bold black bonds indicate bonds formed by radical coupling during lignification; gray bonds result from post-coupling internal rearomatization reactions;  $\alpha$ -OH groups resulting from nucleophilically added water assume the colors of their parent structure. Note that each of these structures represents only one of millions of isomers [2]. Detailed information on these models is given as supplemental material (<http://ars.usda.gov/Services/docs.htm?docid=10443>).

field-grown transgenic trees, have demonstrated that lignin engineering can be beneficial for pulping [3,8,17,58,59]. Knowledge from the monolignol biosynthetic pathway has also been exploited in breeding programs. The most comprehensive example is that of the loblolly pine (*Pinus taeda*) *cad-n1* mutant. Pine trees homozygous for this natural *CAD* null-allele have impaired growth, but their wood is more amenable to chemical pulping. In the heterozygous state, however, the *cad-n1* allele is associated with improved height and wood density [60], in itself a remarkable consequence of the deficiency of a monolignol pathway gene that deserves further study. The *cad-n1* allele is rare because it has been detected only in the elite tree in which it was discovered; nevertheless, sequencing alleles from genetically diverse germplasm should allow identification of this type of defective alleles that can then be introduced into breeding programs. Another example is the genetic association

between a splice variant of the *CCR* gene and microfibril angle in eucalyptus (*Eucalyptus* sp.) [61]. In grasses, such as maize (*Zea mays*), the natural brown-midrib (*bm*) mutants with altered lignin are more digestible by cattle, but their application in plant breeding has been hampered by pleiotropic negative effects, such as susceptibility to lodging. Transcript profiling of these mutants has revealed a set of differentially expressed genes, of which many mapped to quantitative trait loci (QTL) for cell wall digestibility, making these genes good candidates for further study through association genetics [50].

As lignin is also one of the most important negative factors in the conversion of plant material to bio-ethanol, and as several model species, such as maize and poplar, have been put forward as second-generation bio-energy crops, the very same plants that have been studied for their potential improvements for pulping efficiency and fodder



digestibility are being re-evaluated for their saccharification potential. Stover of the maize *bm* mutants and wood from transgenic trees downregulated for *CCR* also have improved saccharification potential ([62]; our unpublished results). Analysis of a set of transgenic (and therefore isogenic) alfalfa (*Medicago sativa*) lines with defects in nearly all steps in monolignol biosynthesis has demonstrated that lignin levels correlate with saccharification efficiency, whereas S/G composition had seemingly no strong effects, in agreement with modeling studies [45,63].

## Conclusions

The recent boom in biofuels stimulates lignin research more than ever. Our knowledge of lignin polymerization and structure, combined with that of systems biology approaches, opens up new avenues to rationally design bio-energy crop varieties with improved processing. The systems biology area is just beginning to deliver its fruits. Various aspects, such as protein–protein interactions and fluxomics, have barely been investigated. However, such studies would open up entirely new views on monolignol biosynthesis and its interaction with other metabolic pathways, developmental processes, and environmental cues. Together with transcript and metabolite profiling data and co-expression analysis, such studies might additionally shed light on the isoforms that are specifically involved in developmental lignification versus those involved in defense lignin [64\*\*]. Finally, fine-tuning of the genetic modification will be possible using specific promoters, RNAi constructs targeted to gene family members, and gene stacking, hence avoiding adverse pleiotropic effects.

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